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Executive Director: EUNICE THOMAS MINER

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*SECTION OF BIOLOGY**

ULTRA-THIN SECTIONS FOR THE ELECTRON MICROSCOPY OF TISSUES†

By JAMES HILLIER‡

Modern electron microscopes can have a practical resolving power of the order of 1 millimicron for suitable specimens. In the study of biological systems which are both heterogeneous and three-dimensional, this implies a potential increase by a factor of nearly 10^7 in the available morphological data. It would appear very unlikely that such an increase in information would fail to exert a great influence on the future development of the biological sciences.

At present, however, these statements must be qualified considerably. Biological systems are dynamic systems. There seems to be little scientific reason for expecting that it will ever be possible to use the electron microscope to examine them in their dynamic (*i.e.*, living) state. Thus, while the direct and continuous observation of the behavior of components of living systems provides the fundamental basis for most interpretations in light microscope, it cannot do so in electron microscopy. The difficulties this introduces are particularly apparent in the study of animal cells where no two cells, identical on a biological basis, can be expected to have the same morphology at the electron microscope level. By taking into consideration the mechanics of electron microscope observations, one is led to the conclusion that interpretation of electron micrographs must be made on the basis of the presence or absence of characteristic and hence recognizable structures and that some significance can be placed on such observations only by correlation with related biological experiments.

Existing techniques for biological specimens provide a considerably reduced increase in information. In addition, that increase varies widely from specimen to specimen and even from point to point within a single specimen. Technically, this is related to the production of contrast in the electron microscope image. It is well known that differences in mass-density (mass per unit area) in the specimen plane produce differences in the scattering of the electrons and that these, in turn, produce the differences in intensity (*i.e.*, contrast) which constitute the image. If now we accept 10 per cent as a conservative value for the minimum contrast required for detection, it can be shown easily that a 1 millimicron sphere placed on a uniform membrane of the same density would not be visible if the membrane were thicker than 10 millimicrons, or that a 1 millimicron sphere embedded in a 10 millimicron thick membrane would not be visible unless its density were one half or double that of the membrane. Obviously, if the full resolving power of the electron microscope is to be used in biology, methods of preparing extremely thin specimens must be devised. An even more fundamental reason for using the thinnest possible specimens is a direct result of the high resolving power. It is apparent that very few superposed layers of structure at the 1 millimicron level leads to complete confusion in a two dimensional image. These, rather than the fact that electrons have low penetrating power, are the real reasons for using thin specimens.

In applying the electron microscope to the study of animal or other tissue, these considerations present some very difficult problems, for it has become desirable to cut sections 100 times thinner than was previously considered possible. At present, this work has reached the point where sections $\frac{1}{10}$th as thick as ordinary thin sections are entirely practical. Since the

* The Section of Geology and Min eralogy and the Section of Physics and Chemistry did not hold meetings in January.

† This paper, illustrated with lantern slides, was the first of three presented at the meeting of the Section on January 8, 1951.

‡ RCA Laboratories, Princeton, New Jersey, and Sloan-Kettering Institute for Cancer Research, New York.

procedure has been described in detail in the literature, it will be outlined only very briefly in this summary.

A conventional microtome using the inclined plane principle of advance can be easily modified to provide theoretically the desired advance (0.2 micron in our case). To achieve this in practice, however, it is necessary to pay special attention to problems of static friction, internal and external vibrations, and variations in temperature. When this has been done, advances of 0.2 micron have been achieved which are both accurate and reproducible to approximately 10 per cent. By arranging a liquid surface with a meniscus that ends at the cutting edge of the knife, it is possible to support and spread the sections as they are cut. This technique also provides the means for obtaining serial sections, which are essential both for the research and for the control of the sectioning technique.

At present, two embedding media have been found satisfactory for such thin sections. The first is the conventional double embedding of collodion and paraffin. The second is the polymerization of n-butyl methacrylate after infiltration of the tissue with the monomer. The second leads to thinner sections, but, at present, is suspected of producing localized but serious distortions of the tissue. Two types of knives have been found satisfactory. The first is a conventional microtome knife carefully sharpened but not stropped. Recently, the addition of a permanent sharpening back has made it possible to reduce the sharpening time to a few minutes The second is an edge of glass broken in a rather special way. No careful comparison of these two types of edge has been made as yet. The remaining aspect of the technique is the mounting of the section for electron microscope observation. In the early work, it was in this step that most of the artifacts were introduced. Now, the sections are placed in the microscope without any treatment whatsoever.

It has been found that any method which *dissolves* some or all of the embedding invariably results in a rearrangement of the fine scale structure. In the above, this does not mean that the embedding is not removed. After the section is in the microscope, a judicious application of the electron beam causes most of the embedding to be removed by sublimation. Since this is a process that proceeds molecule by molecule, the two phenomena which are responsible for artifacts in the conventional techniques, namely, diffusion and surface tension, are completely eliminated.

The sections obtained at present are normally about 0.2 micron thick. The best resolution which has been demonstrated in their images is somewhat better than 20 millimicrons. Thus, this already implies a 1000-fold increase in the information available. While this is by no means the limit of the electron microscope, it is already large enough to cause difficulties of comprehension. In addition, it must be remembered that even at this stage 50 or more serial sections must be examined before an entire cell can be visualized. Since one does not normally have much control over the direction in which a cell is sectioned, it is readily seen that the set of 50 images constituting the three dimensional picture of a single specific cell is only one set randomly selected from well over a million possible sets. When one considers this, along with the facts that it is not possible to follow the behavior of a specific cell and that the different individuals of biologically identical cells are not likely to have identical morphologies, one realizes the futility of attempting to make "life history" or "dynamic" studies with the electron microscope.

These ideas are here introduced not to belittle the significance of the electron microscope in this field, but rather to caution the reader against accepting without question dynamic interpretations based on electron micrographs alone. In the literature, it is not uncommon to find such descriptions as—an electron micrograph of viruses *being* absorbed, protoplasm *being* exuded, and particles elongat*ing*. In view

of the situation outlined above, this is obviously not the way to use an electron microscope. How delightfully simple the solutions of many problems of cytology would be if these difficulties did not exist.

How, then, does one use the electron microscope in biology? At least two types of use already exist. In the first, the electron microscope is used as a means of checking the validity of an indirect method of determining morphology. A beautiful example is to be found in the work on muscle fibres and collagen, where the structures were computed from small angle X-ray diffraction patterns and then verified by means of the electron microscope. This approach is limited, of course, to materials which possess either a uniform morphology or a periodic structure. Otherwise, no answers are forthcoming from the indirect method.

The second type of use, and probably the more important one, is in the study of heterogeneous structures. In this use, interest in a particular biological system usually arises from previous research. This is followed by the development of a satisfactory technique of specimen preparation and a careful study of the system under question. Almost invariably, some structure is seen which is sufficiently characteristic to be recognizable when encountered again but which cannot be identified on the basis of the picture alone. This initiates a program of biological research which has the aim either of identifying the structure within the pattern of existing knowledge or of extending existing knowledge to make it possible to include the presence of a new structure. Having initiated the research, the electron microscope continues to play a role as a means of measuring or evaluating the results of the research since, until further knowledge is obtained, it remains the only tool for detecting the presence or absence or the difference in the interesting structure.

In the latter use of the electron microscope and in the limitations described earlier, one recognizes a striking parallel with the situation in light microscopy at the time, prior to the advent of phase microscopy, when the information obtainable from fixed and stained slides had far surpassed that obtainable from direct observation of living material. In view of this close parallel, it does not seem unreasonable to expect that the electron microscope will exert a similar influence on the development of biological research in the future.

TECHNIQUES FOR THE PRESERVATION OF THREE-DIMENSIONAL STRUCTURE IN PREPARING SPECIMENS FOR THE ELECTRON MICROSCOPE*

By THOMAS F. ANDERSON†

As is now well known, specimens for study in the electron microscope must be freed of all volatile matter before they can be examined in the high vacuum of the instrument. This condition has constituted a great handicap to the microscope's usefulness, particularly in biology, for it renders necessary the removal of water, which is so essential to biological structure and function.

Dr. Hillier has just described how he and others have succeeded in preserving structure by substituting solid materials for the water and then cutting the imbedded specimen into sections only 0.1 μ thick, thin enough to be studied by electron microscopy. If we wish to study whole cells, however, we should like to remove the ambient liquid without adding other materials to increase their opacity and reduce the contrast in their images. When this

* This paper, illustrated with both conventional and stereoscopic lantern slides, was the second of three presented at the meeting of the Section, January 8, 1951. No abstract has been received of the third paper, entitled "Observations of the Fine Structure of Tissue Cells," by Keith R. Porter.

† Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania. This work was supported by a contract between the Office of Naval Research, Department of the Navy and the University of Pennsylvania (NR-135-197), Physical, Chemical and Biophysical Characterization of Viruses and Virus Systems. Reproduction in whole or in part is permitted for any purpose of the United States Government. The technical assistance of Mr. Carl F. Oster is gratefully acknowledged.

is done by simply allowing the specimen to dry in air, the specimen is grossly distorted by surface tension forces. It is the purpose of this paper to describe a method whereby this distortion can be minimized and three-dimensional structures can be preserved in biological materials.

First, however, it will be instructive to inquire into the reasons for the distortion of specimens during air-drying. Suppose the specimen we wish to examine consists of a droplet in which are suspended some cells like bacteria together with some much smaller objects like virus particles. As the droplet is allowed to evaporate, the surface of the water eventually encounters the cells and, since surface tension exerts relatively enormous forces on tiny objects, it presses them flat. Furthermore, virus particles and particles of debris are pushed along under the miniscus and end up around the edges of the cells, which are the last areas to dry completely.

Only the more sturdy skeletal structures, such as diatom shells and butterfly scales,[1] are able to retain their shapes when dried in air. Insect tracheae do also,[2] but, since they are already filled with air before drying, they are a special case. The tiniest tracheae collapse when dried. More delicate specimens, like red cell ghosts, bacteria, viruses, *etc.*, are pressed solidly into the supporting film.[3] As a result, the contrast in electron microscopic images of such specimens is determined primarily by the residual variations in thickness of the collapsed and distorted specimens. These variations in thickness are particularly well brought out by the technique of shadow-casting.

The freeze-drying method was devised[4] to minimize such drying artifacts, but in general the results have been disappointing, perhaps because of the movement of phase boundaries with their inevitable disrupting tendencies. In freeze-drying, such boundaries move through the specimen twice: first, a liquid-solid boundary moves rapidly in the freezing process, and second, a solid-vapor boundary moves during sublimation of the frozen matrix.

Clearly, the problem before us is this: How may the ambient liquid be removed from a specimen without the passage of a phase boundary through it? The answer is straightforward. Take the ambient liquid around its critical point where it changes imperceptibly into a gas. Then allow the gas to escape. This procedure, which is that employed in this work[5] and which was used by Kistler[6] to prepare aerogels, can best be illustrated by reference to FIGURE 1. This figure depicts the pressure-volume isotherms for carbon dioxide. The pressure is plotted as an ordinate

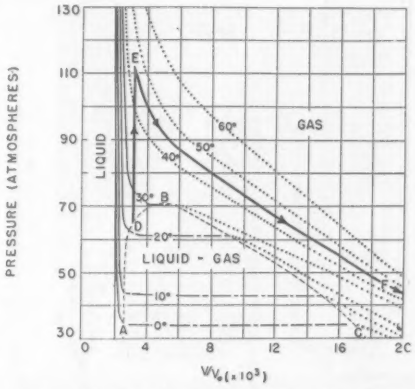

FIG. 1. The path taken by the ambient carbon dioxide during the critical point drying method, plotted on the pressure-volume isotherms of carbon dioxide. Here pressure is plotted as ordinates and the relative volume v/v_0 as abscissae (v is the volume actually occupied by the carbon dioxide in the system, while v_0 is the volume it would occupy at 0°C. and 1 atmosphere).

against the relative volume of the carbon dioxide as the abscissa. Dotted lines indicate gas, solid lines indicate liquid, while dashed lines indicate regions where liquid and gaseous carbon dioxide exist together in equilibrium. It is seen that at temperatures above 31° there is no region in which the two phases can co-exist. This temperature of 31° is called the critical temperature of carbon dioxide. (Diagrams of this sort have been obtained for many substances, each of which has a critical point. This point is related to the attractive forces between the molecules. For example, helium, in which these at-

tractive forces are very small, has its critical point at −268°C., while that for water, in which these forces are great, is at 374°C.) Our method of drying specimens, then, is the following:

(1) The specimen, in a droplet on a formvar-coated screen, is fixed in osmic acid vapor.

(2) Its water is replaced by absolute alcohol by taking it through a series of increasing concentrations in the standard manner. In a similar way, the alcohol is replaced by amyl acetate.

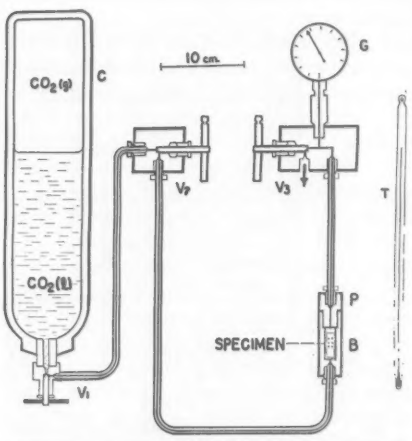

FIGURE 2. The apparatus used in the critical point method. The specimen, S, is placed in the bomb, B, attached by high pressure tubing to valves V_1, V_2, and V_3 and to the inverted tank T containing liquid carbon dioxide.

(3) The specimen in amyl acetate is placed in a bomb attached by high pressure stainless steel tubing to a tank containing liquid carbon dioxide, as shown in FIGURE 2. The bomb is flushed out at 25° with liquid carbon dioxide to replace the amyl acetate by liquid carbon dioxide.

(4) With the bomb completely filled with liquid carbon dioxide, and sealed with the valves V_2 and V_3, the temperature is raised to 35°. During this warming process, the state of the system can be represented by a point moving along the heavy line DE in FIGURE 1.

(5) The escape valve V_3 is gently opened to allow the now gaseous carbon dioxide to blow off. (During this process the state of the ambient carbon dioxide follows the heavy solid line EF out to a pressure of one atmosphere.) Now the specimen is ready for examination in the electron microscope without having suffered the damaging passage of a phase boundary through it.

The consequences of drying biological specimens by this method can best be visualized by viewing stereoscopic pairs of electron micrographs like those shown in FIGURE 3. Stereo views like these are made by tilting the specimen between exposures and they can be seen through an old-fashioned stereoscope. The pair at the top shows a hemolysed human red cell which has retained its spherical shape, except where it has made contact with the supporting formvar membrane, and has fused with it over an area encircled by a dark ring.

The center pair of pictures in FIGURE 3 is a negative print of a discharged trichocyst of *Paramecium aurelia*, together with some spirilla which were present in the culture. It is seen that the trichocyst is cylindrical in cross section and made up of alternate discs of high and low electron-scattering material. The microorganisms can be seen to have retained their cork-screw shapes, as well as certain three-dimensional details of their internal structures. Their flagellae can also be seen stretched in space between various parts of the specimen.

The bottom pair is a stereoscopic view of a bacterium, *Escherichia coli* strain B, sandwiched between two formvar membranes to prevent its escape during the drying process. Some T2 bacteriophage particles can be seen attached to the cell by their tails, and standing out from it like pins on a pincushion. Even the empty head-membranes of some of the phage particles, which are only 0.08 μ in diameter, have not collapsed but retain the straight edges and sharp corners characteristic of the normal particles.

These three examples will suffice to show

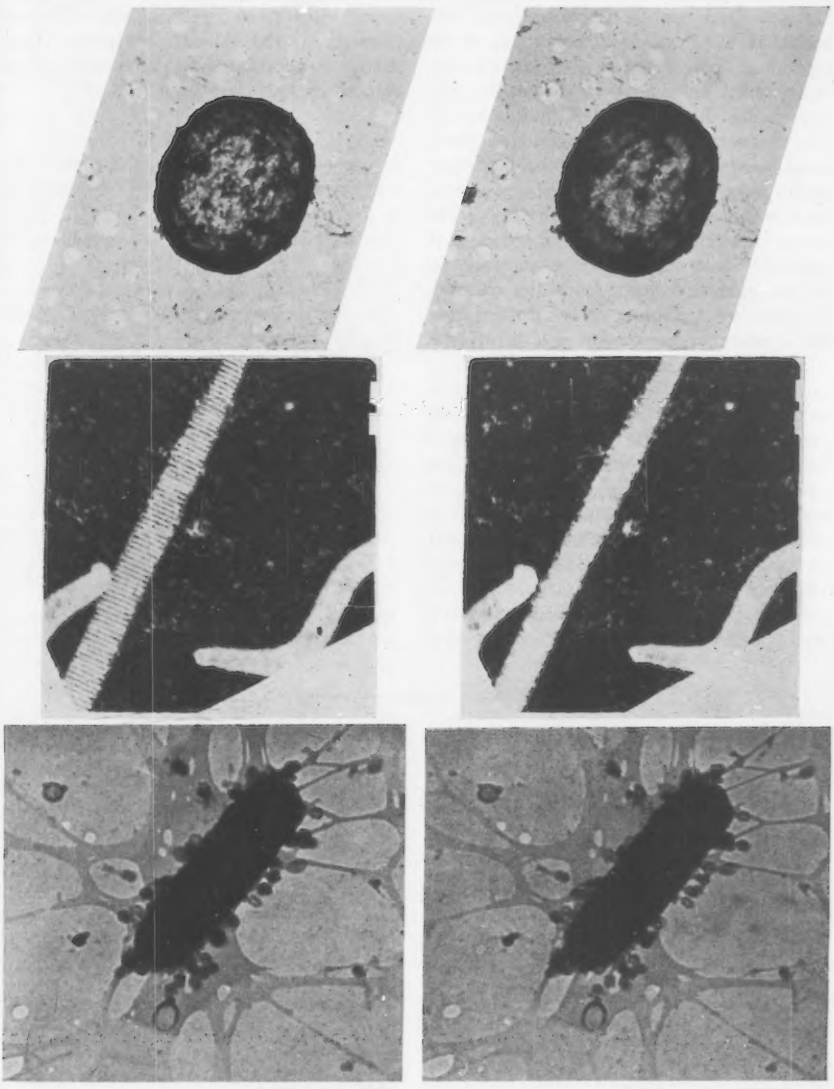

FIGURE 3. (*Top*) A 12° stereoscopic pair of electron micrographs of a hemolysed human red cell which has been dried by the critical point method. It is seen to have retained its spherical shape except where it touches the supporting formvar membrane. ×5,000. (*Middle*) A 12° stereoscopic pair of negative prints of electron micrographs of a part of an extended trichocyst of *Paramecium aurelia* and some spirilla dried by the critical point method. They have retained their shapes. ×7,000. (*Bottom*) A 12° stereoscopic pair of electron micrographs of a bacterium, *Escherichia coli* strain B, on which T2 bacteriophage particles are adsorbed. ×27,000.

that by the critical point method the ambient fluid can be removed from biological systems while still preserving many aspects of their structures. We still have not avoided all distortions, however. For one, the substitution of other liquids for the water must result in the extraction of many constituents. For another, we have already seen the tendency of the specimen itself to reduce its own surface area in the partial flattening of the red cell ghost against the membrane which supports it. As we gain experience with the method, however, we shall hope to learn to minimize and correct for these sources of error, too.

Summary. When specimens are dried for study in the electron microscope, the surface tension of the evaporating liquid produces gross distortions of their structures. It is shown that these distortions can be eliminated by raising the temperature of the ambient liquid above its critical point where, as it loses the capacity to form two fluid phases, its surface tension vanishes. While in this condition, the ambient fluid is allowed to escape, leaving the specimen behind. Stereoscopic electron micrographs of human stroma, bacteria, viruses, *etc.* prepared in this way show that these delicate specimens retain many aspects of their three-dimensional structures.

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*SECTION OF MATHEMATICS AND ENGINEERING**

TUBES FOR MILLIMETER WAVES†

By J. R. PIERCE‡

Electromagnetic radiation is an inexhaustible natural resource which affects a great deal of our life and our technology. In connection with our technology, we are most apt to think of electromagnetic waves in connection with radio. We describe radio waves by using two terms: wavelength and frequency. The wavelength is simply the distance from crest to crest as the wave travels through space. The frequency is the number of crests which pass a given point in a second. As all electromagnetic waves travel with the velocity of light, the number of crests which pass per second is the velocity of light divided by the distance from crest to crest; that is, the frequency is the velocity of light divided by the wavelength.

Frequency is important because the range of frequencies used in transmission is a measure of the complexity of the message which can be sent. Thus, a telephone conversation requires a band of frequencies only 4,000 cycles wide, but television requires a band about 1,000 times as wide. Consider a given wavelength of radio waves, say, 100 meters, corresponding to a

* The Section of Psychology held a meeting on January 15th at which Harry Helson presented a paper entitled "An Experimental-Theoretical Approach to Adaptive Behavior." No abstract of this paper has been received.

† This paper, illustrated with lantern slides, was presented at the meeting of the Section on January 19, 1951.

‡ Bell Telephone Laboratories, Murray Hill, New Jersey.

frequency of 3 megacycles. It is clear that the wavelength range from 50 meters to 100 meters affords as broad a band of frequencies as the range from 100 meters to infinity. Thus, shorter wavelengths afford more frequency band width and enable us to send more and more complicated messages.

Wavelength is important because it is related to the size of apparatus used in generating radio waves and in launching them into space. To be most efficient in launching and directing radio waves, antennas should be large compared with the wavelength. To be most efficient in generating radio waves, electron tubes should be small compared with the wavelength.

Let us consider some examples. A typical AM broadcast wavelength is 400 meters (750 kilocycles). For this wavelength, it would be hard to make an antenna many wavelengths tall. The antennas used are usually towers ¼ wavelength tall (330 feet tall). They radiate signals in all directions (except up). On the other hand, tubes for AM broadcast use are much less than 400 meters in size, and they are very efficient.

The center of the lower-frequency television band is around 4 meters (70 megacycles). The half-wave receiving antennas which are sometimes used are about 6½ feet wide. Transmitting antennas are much larger and send out waves over a narrow vertical angle only. Vacuum tubes are much smaller than 4 meters, and conventional tubes are still efficient at this wavelength.

The center of the higher frequency television band is around 1.5 meters wavelength (200 mc.). Some of the older television masts have a half-wave antenna about 2½ feet wide to receive this band, as well as a 6½ foot antenna for the lower frequency television band. At this wavelength, a transmitting antenna can easily be many wavelengths tall, and hence it can be highly efficient and directive. Although conventional tubes are small compared with 1.5 meters, at this wavelength their performance is beginning to fail.

Microwave radio is used in radar and to send telephone and television for long distances in a series of line-of-sight hops. For instance, the American Telephone and Telegraph Company's TD-2 system, which carries television programs to many cities in the Middle West and which will eventually span the country, uses a wavelength of 7.5 centimeters, corresponding to a frequency of 4,000 megacycles. The antennas used are 10 feet square, which is many wavelengths, and they produce a narrow beam of radio waves directed at the receiver. The broad receiving antennas intercept radio waves over a large area. Thus, a power of ½ watt sends a television signal 30 miles. Even at this short wavelength, the tubes used are smaller than the wavelength of 7.5 centimeters (3 inches). Nevertheless, although they are of special design adapted for operation at this short wavelength, they are rather inefficient compared with tubes used for lower frequencies.

As one goes from wavelengths of a few centimeters to wavelengths of a few millimeters, tubes of special types must be used in order that the tubes do not become vanishingly small. At shorter and shorter wavelengths, the tubes used become larger and larger compared with the wavelength, and in general tube performance declines.

All men, through sight and touch, and physicists through spectroscopy, utilize a different part of the electromagnetic spectrum. Yellow light has a wavelength of 5.8 ten-thousands of a centimeter and a frequency of about 500 million megacycles. In their studies of the fundamental properties of matter by spectroscopy in the far infra-red, physicists have reached wavelengths of around one millimeter (a frequency of 300,000 megacycles), but only with extreme difficulty.

FIGURE 1 gives some idea of the troubles encountered. The horizontal scale at the top shows wavelengths in millimeters. At the extreme left is a wavelength of 1/100

millimeter, in the far infra-red. At the extreme right is a wavelength of 100,000 millimeters or 100 meters, which is about ⅓ the wavelength used in AM broadcast.

The vertical scale at the left shows power in watts, ranging from 100 million watts at the top to a millionth of a millionth of a watt at the bottom.

The lines in the lower left show the power radiated from a black body of 1 square centimeter area heated to 5,000°K. which corresponds roughly to the hottest infra-red sources available. The lower

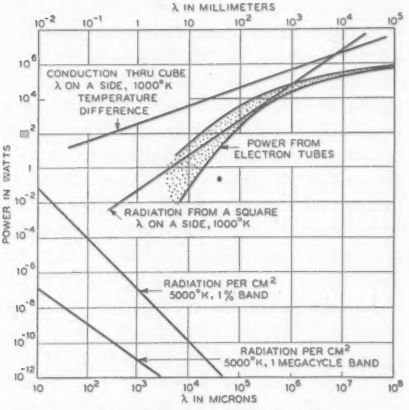
λ IN MILLIMETERS
10⁻² 10⁻¹ 1 10 10² 10³ 10⁴ 10⁵
CONDUCTION THRU CUBE λ ON A SIDE, 1000°K TEMPERATURE DIFFERENCE
POWER FROM ELECTRON TUBES
RADIATION FROM A SQUARE λ ON A SIDE, 1000°K
RADIATION PER CM² 5000°K, 1% BAND
RADIATION PER CM² 5000°K, 1 MEGACYCLE BAND
POWER IN WATTS
10⁶ 10⁴ 10² 1 10⁻² 10⁻⁴ 10⁻⁶ 10⁻⁸ 10⁻¹⁰ 10⁻¹²
10 10² 10³ 10⁴ 10⁵ 10⁶ 10⁷ 10⁸
λ IN MICRONS

FIGURE 1. The vertical scale shows power and the horizontal scale, wavelength.

curve is the power in a band of frequencies 1 megacycle wide; the upper curve is the power in a band of frequencies 1 per cent wide. We see that the power available from a hot source falls rapidly as the wavelength is increased.

The dotted region in the upper right roughly encompasses the average power available from electron tubes. We see that this declines sharply with decreasing wavelength, falling from a maximum of around a megawatt at broadcast wavelengths to around one watt at a wavelength of 3 millimeters, which is about the shortest wavelength reached so far with electron tubes.

At very short wavelengths, hot bodies such as the filaments of incandescent lamps are admirable sources of electromagnetic waves. At long wavelengths, vacuum tubes are both easy to make and efficient. The millimeter range is a depressed region in the production of electromagnetic radiation, yet potentially it is an important one. Physicists feel that they can learn much through millimeter-wave spectroscopy. To technology, the millimeter region offers almost limitless frequency bandwidth to carry our expanding communications. It also offers the possibility of sending radio signals long distances through small pipes or waveguides.

None of these potentialities can be realized without adequate means for generating and perhaps for amplifying millimeter waves. And, to the electron tube man, the millimeter range is a headache.

The fundamental reason for this is clear. Tubes work best when they are small compared with a wavelength. Small tubes are hard to build. Worse than this, the smaller a tube is made the harder it is to keep it cool. The upper straight lines illustrate this. The top line shows thermal power conducted through a copper cube a wavelength on a side with a temperature difference of 1,000°Kelvin. The lower line shows power radiated from a black area a centimeter square at a temperature of 1,000°Kelvin. It is no coincidence that these curves slant in the same direction as that showing the power output which has been attained using electron tubes.

There are other difficulties in the way of making millimeter wave tubes as well. For one thing, to get a given power at a given voltage, one requires a certain current. As a tube is made smaller and smaller, the current density which must be drawn from the cathode increases, varying inversely as the area of the cathode and hence inversely as the square of the length or width. If the tube dimensions are to be roughly proportional to the wavelength, the cathode current density required tends to vary inversely as the square of the wavelength. Now, a given cathode material at a given

temperature emits only so many amperes per square centimeter. Hence, in making tubes smaller and smaller, there comes a point at which one simply cannot obtain the desired current density from the cathode.

The fact that electrical losses in metal tend to vary inversely as the square root of the wavelength tends to degrade the performance of millimeter wave tubes, making them lossy and inefficient.

Summarizing in order of importance the troubles in making tubes for millimeter wavelengths, we might list them: (1) problems of heat dissipation from parts of small area and cross-section; (2) problems of cathode current density; (3) increase of electrical losses at short wavelengths; and (4) difficulty of fabricating small structures.

How are these difficulties overcome? Let us consider one type of tube which has been used successfully in the millimeter range—the pulsed magnetron.

The pulsed magnetron was the glamor tube of World War II, the tube which made microwave radar practical. At a wavelength of 10 centimeters, for instance, a magnetron can generate bursts of radio frequency energy at powers of several million watts.

Why is the pulsed magnetron so successful? Let us compare the magnetron with a triode. In a triode, the essential feature of operation is the control of the electron current from a flat cathode by a voltage applied to a grid. The grid is made of fine parallel wires, and it must be close to the cathode. In a typical triode used for wavelengths around 10 centimeters, the grid is made of wires $\frac{1}{3,000}$ of an inch in diameter, spaced 1,000 to the inch, and the spacing between the grid and the cathode is about $\frac{1}{2,000}$ of an inch. Contrast this structure with that of a pulsed magnetron.

FIGURE 2 shows the essential parts of a magnetron: a central cathode surrounded by a copper anode in which there are cut a number of resonant elements or slots. In operation, the anode is made positive with respect to the central electron-emitting cathode. A magnetic field perpendicular to the plane of the paper causes the cloud of electrons between the anode and the cathode to rotate. The radio-frequency electric fields of the anode slots tend to form the

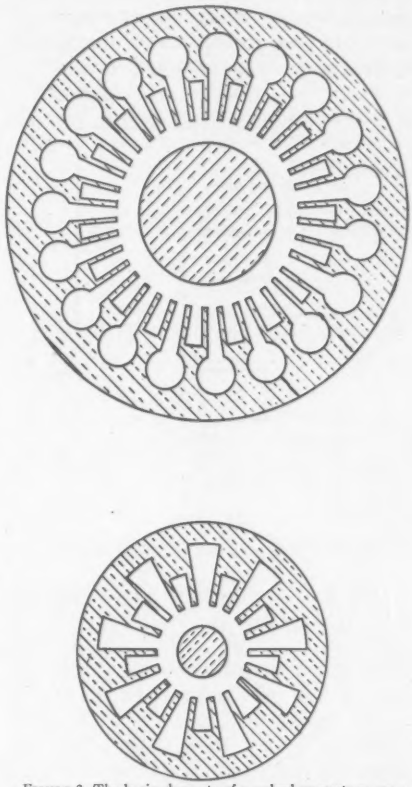

FIGURE 2. The basic elements of a pulsed magnetron are a central cathode and a surrounding anode into which are cut slots or resonators. The anode is made positive with respect to the cathode. A magnetic field is applied normal to the plane of the illustration.

electron flow into rotating spokes of charge, and these spokes induce currents in the resonators and transfer power to the radio-frequency field.

Not only is the anode of a magnetron strong compared with the flimsy grid of the triode, but the spacing between cathode and anode in a magnetron for a 10 centimeter wavelength is several hundred times

as great as the spacing between the cathode and grid of a triode for the same wavelength. Thus, the magnetron is larger, stronger, and more rugged, and is capable of dissipating more heat than a triode. This explains why the magnetron can give peak powers of several megawatts, while the triode gives steady powers of around a watt.

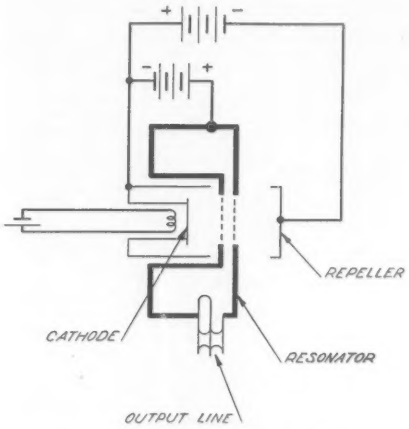

FIGURE 3. The chief elements of a reflex klystron.

Why is the magnetron so large and powerful? It is partly due to the principle of operation. In the triode, the electrons, which move comparatively slowly between cathode and grid, must make the trip or transit in a small fraction of a radio-frequency cycle. In a magnetron, comparatively fast electrons take several cycles to go from cathode to anode; hence the disparity of spacings.

Partly, however, the magnetron is large and powerful because it is used for pulsed operation. Operation at several tens of thousands of volts allows a wide spacing between cathode and anode. The current density which a cathode can supply for short periods is large compared with that which it can supply continuously. Furthermore, tubes work most efficiently when they are driven hard. Large and rugged as it is, the magnetron cannot run continuously at a megawatt level, but it can run at this level for a thousandth of the time.

Thus, two secrets of making large, rugged tubes for a given wavelength are (1) to choose a tube which, like the magnetron, is naturally large and (2) to run it pulsed, intermittently at higher voltages and currents than those at which it could run continuously.

The highest peak and average powers which have been achieved in the millimeter range have been generated by pulsed magnetrons. Workers at Columbia University have obtained 30–40 kilowatts at 6 millimeters, 5–15 kilowatts at 4.5 millimeters, and 1–2 kilowatts at 3 millimeters. A small power at a wavelength of 1.45 millimeters has been obtained as a harmonic from a tube operating at 4.35 millimeters.

Pulsed power is not suitable for some uses. When one goes to continuous operation and sacrifices the advantages of high power and large physical size, magnetrons become less satisfactory. Several other types of tubes have been used for the steady generation of small amounts of power in the millimeter range. One of these is the reflex klystron, illustrated schematically in FIGURE 3.

In the reflex klystron, an electron stream from a cathode is drawn across the center of a doughnut-shaped resonator. A radio-frequency voltage appears between two opposed grids through which the electrons pass. This voltage alternately speeds up and slows down the electrons. The electrons are repelled and returned through the resonator by a negative repeller electrode. Now, the electrons which have been speeded up penetrate the retarding field further toward the repeller, and take longer to return. Hence, the initially constant-current stream becomes bunched by the time it returns through the resonator. The repeller voltage is so adjusted that the returning bunches encounter a retarding field in passing across the resonator, and hence they give up energy to the electric field.

The reflex klystron is superior to the

triode for operation at very short wavelengths chiefly because the electrons pass across the gap between the grids at a high speed. They must make this transit in a small part of a cycle, but they are going fast. Hence, while the cathode-grid spacing for a triode designed to operate at 7 centimeters is around $\frac{1}{2.000}$ of an inch, the spacing between the grids of a reflex klystron which operates at 7 millimeters is around $\frac{1}{300}$ of an inch. Reflex klystrons have been built which give a few thousandths of a watt at wavelengths around 5 millimeters.

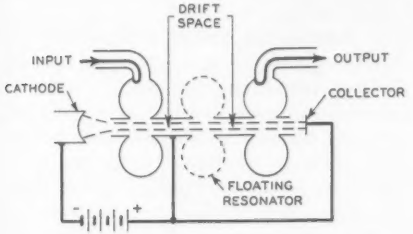

FIGURE 4. The chief elements of a klystron amplifier.

Sometimes we wish not merely to generate signals of millimeter wavelength, but to amplify them as well. Except for the triode, none of the devices mentioned so far is capable of amplification, but the klystron is (as contrasted with the *reflex* klystron, which is actually a later development).

FIGURE 4 is a schematic representation of a klystron. An electron stream from a cathode passes across a doughnut-shaped input resonator. A voltage across the resonator periodically speeds up and slows down the electrons. The electrons then pass through a drift space in which the fast electrons catch up with the slow electrons, and the electron stream becomes bunched. Finally, the bunched electron stream passes through an output resonator and its fluctuating current induces an amplified signal in this resonator. A greater gain can be achieved by use of a third "floating" resonator placed between the input and output resonators.

Klystrons work admirably as amplifiers down to wavelengths of 3 centimeters, and they can be made to amplify down to around 1.25 centimeters.

A newer and perhaps more promising amplifier for millimeter waves is the traveling-wave tube. Some virtues of the traveling-wave tube are physical simplicity and high gain.

FIGURE 5 shows the essential elements of a traveling-wave amplifier. They are an electron stream and a circuit which will carry a slow electromagnetic wave. One

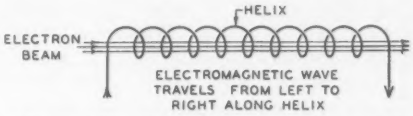

FIGURE 5. The basic elements of a traveling-wave tube are an electron stream and a circuit which will carry a slow electromagnetic wave.

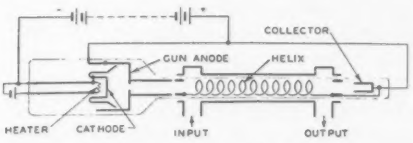

FIGURE 6. The parts of a traveling-wave tube.

such circuit which is much used in traveling-wave tubes is a helix or coil of wire. It should be so proportioned that the electromagnetic wave travels with nearly the same speed as do the electrons, and in the same direction.

When this is so, it is found that a signal put on the helix increases with distance, and finally exponentially with distance. The helix plus electron stream has a negative attenuation. Physically, the field of the wave acts to bunch the electron stream, and the bunched electron stream in turn gives up power to the wave. For this to take place, the electrons must travel a little faster than the wave. We may liken the action to that of a wind (the electrons) blowing past the ripples on a pond (the electromagnetic wave) and causing small ripples to grow into large waves.

FIGURE 6 shows the parts of a traveling-wave tube: the electron gun, consisting of

a cathode and a positive anode to accelerate the electrons; the helix; input and output metal tubes or wave guides to lead the wave to and from the helix; and a collector electrode to collect spent electrons.

J. B. Little of the Bell Telephone Laboratories has built a traveling-wave tube capable of amplifying a signal of 6–7 millimeters wavelength. The helix in this tube is of wire $\frac{3}{1,000}$ of an inch in diameter, with an inside diameter of $\frac{3}{100}$ of an inch. The input and output wave guides have openings $\frac{3}{16}$ of an inch by $\frac{3}{32}$ of an inch. The tube requires a current of 1 milliampere at 1,000 volts. It gives a power gain of around 3 times.

More rugged traveling-wave tubes, with circuits other than helices, have given gains of 10 times and powers of around $\frac{1}{100}$ of a watt at a wavelength of 6 millimeters.

Beyond what has been done, there have been a variety of suggestions for generating millimeter waves. A type of amplifier called the electron-wave or double-stream tube employs two intermingled electron streams instead of an electron stream and a circuit, as does the traveling-wave tube, and achieves results similar to those obtained with the traveling-wave tube. Electrons traveling faster than light (they can do this only in a dielectric, in which electromagnetic waves are slowed down) radiate electromagnetic energy, and this Cherenkov radiation has been suggested as a source of millimeter-wave power. Electrons accelerated from side to side radiate, and it has been suggested that an electron stream be shot through the fields of a series of alternately poled magnets to achieve a sinuous motion. The doppler effect causes the forward radiation from such a sinuous stream to have a higher frequency than the actual frequency of oscillation of the electrons from side to side.

At present, the middle of the millimeter range has been reached with experimental electron tube oscillators and amplifiers. Certainly, better tubes will be made, and tubes for higher frequencies. In the field of pure science, we may expect advances in millimeter-wave spectroscopy to follow and to shed new light on the constitution of matter. In the field of technology, we may expect the range of millimeter waves to provide us with channels for long and short distance communication.

*DIVISION OF MYCOLOGY**

PHYSIOLOGICAL BASES OF MORPHOGENESIS IN ANIMAL DISEASE FUNGI†

By WALTER J. NICKERSON‡

Most of the fungi causing disease in animals and man exhibit morphological aspects in culture which are not observed *in vivo*. Many of the organisms responsible for deep mycoses are found as yeasts in tissues. *In vitro* they can be experimentally controlled to exhibit either a yeast or a mycelial form. The dermatophytes commonly sporulate only rarely *in vivo* but may do so abundantly on appropriate solid media. The physiological bases of morphogenesis have been examined for some of these organisms. Study of these problems has contributed information on the chemical control of morphogenesis and the mechanism of cell division and may shed some light on the extraordinarily complex problem of the mechanisms of pathogenesis in mycotic infection.

Temperature Control of Morphogenesis in

* The Section of Anthropology held a meeting on January 22nd at which a paper entitled "American Indian Warfare" was presented by Marian W. Smith. No abstract of this paper has been received.

† This paper, illustrated with lantern slides, was presented at the meeting of the Division of Mycology on January 26, 1951. The research for this paper was aided in part by a grant from the American Cancer Society made upon the recommendation of the Committee on Growth of the National Research Council.

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Blastomyces. The *in vitro* conversion of mycelial-type growth to a yeast-type of growth has been shown to be independent of nutrition and regulated only by temperature in *Blastomyces dermatitidis*[1–4] and in *Blastomyces* (*Paracoccidioides*) *braziliensis*.[3,4] The intracellular physicochemical environment developed at 37° allows morphogenesis of the yeast form in these two species, while that developed at 25° permits only the mycelial form. This dimorphism has been analyzed[2,3] to result from the failure of the cell environment to maintain the necessary yeast-type rate in cell division processes at the lower temperature, with the consequence that growth of *Blastomyces* in length and volume (growth proceeding only somewhat slower at 25° than at 37°) is no longer maintained in balance with the cell division process. The exact manner in which this temperature effect operates to alter the physico-chemical environment of the cell is not known, but it has been learned that the enzyme system responsible for exogenous respiration in *Blastomyces* is developed only at the higher incubation temperatures.[3] The yeast-forms of these two species have the property of transforming the energy of their substrates 5–6 times faster than the rate per unit weight in mycelial forms.

Role of Sulfhydryl (—SH) Group in Morphogenesis of Candida. With other pathogenic fungi exhibiting both mycelial and yeast forms, temperature has been found either not to be a controlling factor or not to be the only controlling factor. In studies using well-defined culture media, the importance of sulfhydryl group compounds for the maintenance of the yeast form in *Candida*[2,5] and in *Histoplasma capsulatum*[6] has been shown. With *Candida albicans*, we have recently found[7] that the effect of glucose metabolism on the maintenance of the yeast form (thereby preventing the appearance of the mycelial form) operates through the intracellular production of sulfhydryl (—SH) groups at the lowered redox levels obtained during glucose metabolism. The mechanism of the sulfhydryl effect on cell division processes is not completely known. We have evidence from parallel work on *Candida albicans* and on *Bacillus mesentericus* (chemical analyses on the normal, small rods and on masses of greatly elongated cells) that (—SH) is required, not for the synthesis of nucleic acids, but apparently only for the polymerization of nucleic acids.[8]

Recently, we acquired from Dr. Juan E. Mackinnon some mutant strains of *Candida albicans* which are filamentous under conditions where normal strains are only in the yeast form. These mutant strains were described by Mackinnon[9] as the so-called "rough" forms. By adding 10^{-2} M cysteine to a glucose-glycine-yeast extract medium, the mutant strain 806 has been obtained largely in the yeast form, while the control, without an external source of —SH, grows largely in a filamentous condition (FIGURE 1). These two types of growth have been examined in different ways. Their content and their localization of alkaline monophosphatase are similar, and their respiratory patterns are apparently identical[14] (TABLE 1).

In view of the dependence of normal strains of *Candida albicans* on exogenous glucose metabolism for maintenance of (—SH) and the yeast shape, it was deemed of interest to learn whether addition of (—SH) to strain 806 would result in an increase in the rate of glucose oxidation (certain of the enzymes involved being known to be sulfhydryl-containing enzymes). From this result (TABLE 1), one may infer that the specific importance of glucose oxidation for cell division processes (as distinquished from its importance for growth processes) lies in the formation of reduced (—SH). This finding is actually only a confirmation of our previous report[7] of the successful maintenance of the yeast form in normal strains of *Candida albicans* on polysaccharide media containing 10^{-2}M cysteine but no readily metabolizable carbon source.

Cytochemical localization of sulfhydryl groups in the cells of strain 806 grown in

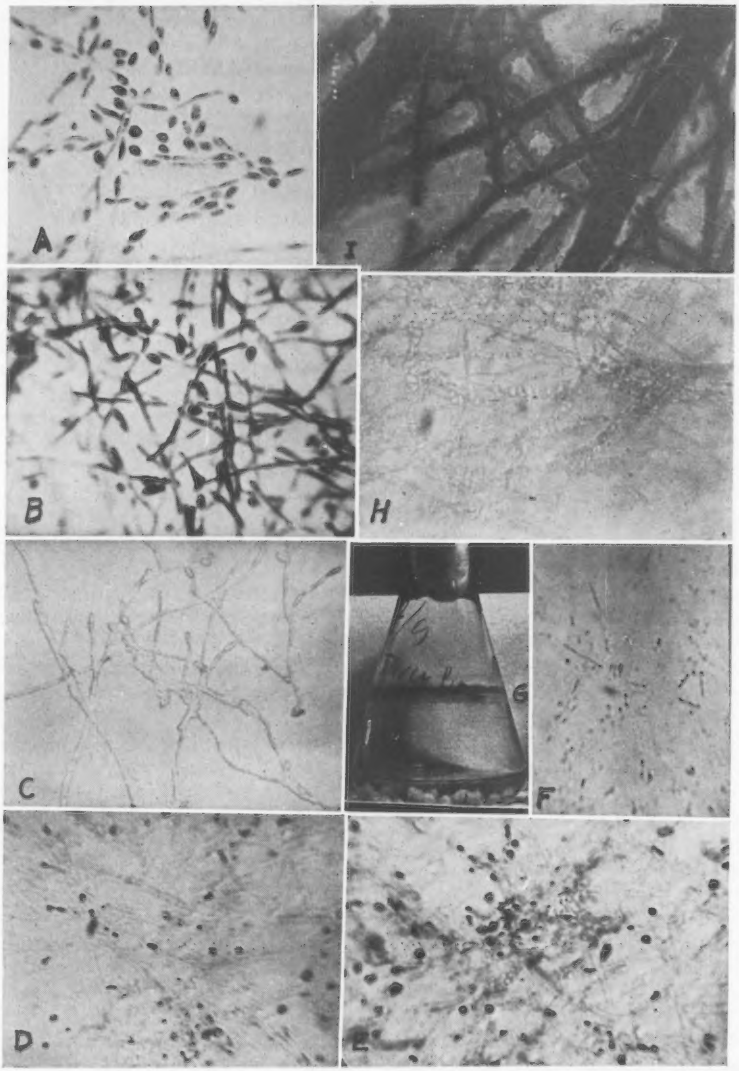

FIGURE 1. A–F, *Candida albicans*, strain 806: A–C showing alkaline phosphatase localization by Gomorri technique with sodium glycerophosphate as substrate. Cells in "A" grown with added cysteine (10^{-2} M), without such addition in "B" and "C." "C" is of heated control. "D–F" show localization of sulfhydryl group centers by means of Prussian blue reaction of Chèvremont and Frederic; "D" and "E" show high concentrations of —SH (mainly in yeast-type cells) in cells grown with added cysteine; cells in "F" grown without added cysteine.

G–I, *Trichophylon rubrum*: flask in G contains culture grown under continuous shaking for six days; small spheres visible in medium and band type growth visible half way up the flask. Alkaline phosphatase localization (sodium glycerophosphate) shown in "I," with heated control in "H."

the presence, or absence, of added cysteine was studied by the technique of Chèvremont and Frederic.[11] In this procedure, the cells are treated with a strong solution of trichloracetic acid to expose —SH in the cell. The —SH is then reacted with ferricyanide, whereby Prussian blue is formed, giving bright blue spot localizations for —SH centers. The cells grown with added cysteine showed numerous, dense —SH centers, especially in the yeast-type cells, while the cells grown without added cysteine showed a more infrequent, less dense distribution of —SH with mycelial type cells practically devoid of —SH centers (FIGURES 1D–F).

TABLE 1

EFFECT OF CYSTEINE ON GROWTH AND RESPIRATION OF ROUGH STRAIN OF *Candida albicans* (806)*

Culture	*Turbidity (Klett units)*	*pH of culture*	*Oxygen uptake endogenous* Q_{O_2}	*Oxygen uptake exogenous* Q_{O_2}
Control	390	5.4	30.0	33.1
$+ 10^{-2}$ M (—SH)	420	6.5	31.8	32.0

* Grown in glucose-glycine liquid med um at 32° in shaken cultures for 23 hours.[14]

Metabolism and Morphogenesis of Dermatophytes in Shaken Cultures. With the dermatophytes, there is only a mycelial form of growth in culture, but sporulation in culture can be enormously greater than ever observed *in vivo*. In order to obtain larger quantities of cell material for studies on the proteolytic enzymes of *Trichophyton*, we have grown *T. mentagrophytes* and *T. rubrum* in liquid media in shaken cultures.[10] It is, of course, not new to grow fungi in shaken cultures (the method was used to obtain uniform growths of *Blastomyces* for respiratory studies[3]), but the method does appear to have been untried before with dermatophytes.

The two species studied grew abundantly as small pellets (FIGURE 1) of relatively uniform diameter (TABLE 2) that have proved to be most convenient to handle for manometric studies and in chemical investigations. Burkholder and Sinnott[12] showed that many filamentous fungi grow as spheres of characteristic size in shaken cultures. In addition to the growth as spheres, our cultures developed a band around the upper portion of each flask. This band (FIGURE 1) presented the appearance of growth commonly obtained on agar media. It was fluffy and composed of pigmented vegetative mycelium and spores, in contrast to the small spheres, which were composed of compact, nonpigmented mycelium devoid of all spores except for a few thick-walled, intercalary spores. The submerged growth of *Trichophyton* is thus much like the growth of these fungi *in vivo*.

TABLE 2

PHOSPHORUS FRACTIONS OF *Trichophyton* GROWN IN SHAKEN CULTURES[14]*

Species	*Diameter of mycelial balls*	*Average dry wt./ball*	*Cold acid soluble P µg./mg. dry wt.*	*Hot acid soluble P µg./mg. dry wt.*
Trichophyton mentagrophytes	2–3 mm.	0.49 mg.	1.6 1.3	2.4 2.3
Trichophyton rubrum	5–6 mm.	4.0 mg.	1.3 1.2	0.9 1.0

* Incubated 6 days at 30 C. in glucose-peptone liquid medium.

Some aspects of the composition and metabolism of the submerged growth and of the aerial growth have been examined. Manometric determination of oxygen consumption by the spheres of both species showed them to have the same rate of respiration as was found earlier[13] for these species grown on agar media. With neither material was an exogenous oxidation of glucose detectable. In both studies, the Q_{O_2} for *T. rubrum* was lower than for *T. mentagrophytes*.

The phosphorus contents of fractions extractable from the submerged growths by cold trichloracetic acid and by hot trichloracetic acid (after lipid removal)

were estimated (TABLE 2). The phosphorus in the hot acid extract was determined as a measure of the nucleic acid content of the cells. Pentose determinations (orcinol method) on the hot acid fraction were carried out to measure the pentose nucleic acid extracted (TABLE 3). Desoxypentose nucleic acid (DNA) was also estimated in the hot acid extracts from *Trichophyton mentagrophytes* and from *Trichophyton rubrum*. Only small amounts of DNA have been detected. (The presence of nucleic acids in *Trichophyton rubrum*

TABLE 3

RIBOSE NUCLEIC ACID COMPOSITION OF *Trichophyton mentagrophytes* GROWTH IN SHAKEN CULTURES[14]*

Growth type	*Sample Dry wt. mg.*	*Pentose content μg./mg. dry wt.*	*RNA μg./mg. dry wt.*
Submerged (6 day culture)	18.1	11.4	49.8
Submerged (6 day culture)	20.6	10.7	46.6
Submerged (14 day culture)	23.2	7.7	33.6
Aerial (14 day culture)	22.0	6.5	28.0
Aerial (14 day culture)	25.1	6.8	29.2

* Incubated at 30°C. for time stated in glucose-peptone liquid medium. Values are averages.

was observed during determinations of the absorption spectra of hot acid-ethanol extracts of pigmented cultures grown on agar plates.[10] The characteristic nucleic acid absorption at 2650 Å was marked in these extracts). This is believed to be the first determination of the nucleic acid content of dermatophytes.

Application of the Gomorri[15] technique for localization of alkaline phosphatase of submerged growths of *Trichophyton* (FIGURE 1, H–I) revealed the spheres to be relatively rich in such enzymes. Sodium-β-glycerophosphate, hexose 1,6-diphosphate, and various inorganic poly- and metaphosphates have been employed as substrates at pH 8.9, with tris-hydroxy-methyl amino methane as buffer. Glycerophosphatase is shown to be present in abundance in *Trichophyton rubrum* in FIGURE 1, I. The enzymatic activity of this organism against the substrate is destroyed by heating the cells for three minutes in boiling water (FIGURE 1, H). Few differences have been observed between the phosphatase activity of submerged and of aerial growths thus far.

Summary. The regulating action of temperature on the interconversion of mycelial and yeast types of growth in the dimorphic fungi, *Blastomyces dermatitidis* and *Blastomyces braziliensis* is discussed in relation to the development of an exogenous respiratory system in these organisms incubated at 37°C.

In *Candida albicans*, the importance of intracellular sulfhydryl groups for maintenance of the yeast form is demonstrated. In a mutant "rough" strain with an imbalance in the cell division/growth processes, the addition of —SH to culture media resulted in the production of an almost completely yeast type of growth. The yeast cells were somewhat richer in alkaline phosphatase and much richer in cytochemically demonstrable sulfhydryl groups than the mycelial-like cells, but the culture grown with addition of —SH had almost exactly the same rate of respiration as cells of the culture grown without added —SH. The specific importance of cellular respiration for cell division processes (as distinguished from growth processes) is considered.

Two different species of *Trichophyton* have been grown in cultures subjected to continuous agitation. Nonsporulating compact clumps of mycelia obtained in this manner have been studied in comparison with normal, sporulating growth. Alkaline phosphatase has been shown cytochemically to be present in abundance in the mycelia. Pentose- and desoxypentose-nucleic acid analyses have been carried out on both types of mycelia.

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A NEW HISTOCHEMICAL TOOL FOR THE DEFINITIVE DIAGNOSIS OF FUNGUS INFECTIONS*

By DONALD M. PILLSBURY AND ALBERT M. KLIGMAN†

The diagnosis of a fungus infection may be accomplished definitively by two laboratory procedures. These are: (1) the direct demonstration of fungus cells in biopsy specimens, exudates, tissue fluids, or skin scrapings; and (2) the isolation of the causal organism in cultures. Of these, the direct demonstration method is the most practical under ordinary clinical circumstances. Even in proved fungus infections of the skin such as ringworm of the feet, direct microscopic methods of examination yield positive results more than twice as often as cultural procedures.

In the systemic fungus infections, weeks may be required to isolate the offending organism, and even after this is done specialized knowledge is required to determine the species. The direct examination of accessible tissue samples, however, may permit a definitive diagnosis in a few minutes or in a few days if biopsies are taken. From a diagnostic standpoint, it is perhaps fortunate that the different fungi causing systemic disease have distinctive histopathologic and morphologic characteristics in tissue. That is, if the organism can be visualized at all, it can generally be identified.

Unfortunately, the routine histologic methods for processing tissues are inadequate in so far as demonstration of fungi are concerned. Hematoxylin and eosin, while suitable for general pathologic work, has strong limitations with regards to fungus infected tissue. The organisms stain poorly and sometimes not at all. The organisms are so inconspicuous that they are frequently not visualized unless, on the basis of other data, one strongly suspects fungus infection. Nonetheless, the hematoxylin and eosin stain has probably been the best technique for routine mycologic work.

The potassium hydroxide preparation has stood the test of time as a classical procedure for the demonstration of fungi in the skin and keratinous appendages; that is, in the hair and nails. The organisms most commonly invading the skin and its appendages belong to the ringworm fungi. The undifferentiated filaments of these organisms grow in the dead stratum corneum or horny

* This paper, illustrated with lantern slides, was presented at a meeting of the Division of Mycology on November 24, 1950. The study on which the paper is based was supported by a grant from the Research and Development Board, U. S. Army.

† Department of Dermatology and Syphilology, School of Medicine (Dr. Donald M. Pillsbury, Director), University of Pennsylvania, Philadelphia, Pa.

layer of the skin. The filaments of species belonging to the various genera of the ringworm fungi (*Microsporum*, *Epidermophyton*, and *Trichophyton*) cannot be distinguished. The usual technique of making a potassium hydroxide mount is simply to scrape some of the scales from the peripheral portion of the lesion. These are put into a drop of 10 per cent potassium hydroxide. The strong hydroxide solution serves to clear the keratinous scales by hydrolysis, so as to render the more resistant fungi visible This type of preparation is unstained. It is a temporary one, and some experience is required to recognize the fungus elements. The differentiation of artifacts is sometimes difficult. Occasionally, one may find it necessary to examine a slide for fifteen minutes or more before recognizing a diagnostic fragment of fungus.

It is clear that there are deficiencies in the direct microscopic methods utilized for the diagnosis of fungus disease. In recognition of this fact, a study was undertaken to examine the possibility of finding a histopathologic staining technique which would prove valuable for routine diagnostic work. The periodic acid-Schiff technique was found to be quite suitable.

Rationale of the Stain. The usefulness of the periodic acid-Schiff procedure derives from a knowledge of the chemical structure of the fungus cell wall. In contrast to animal cells, the cell walls of fungi are made up of cellulose and chitin complexes. These materials endow the fungus cell with a certain rigidity and durability. Therefore, staining techniques which visualize cellulose (a complex polysaccharide) are likely to be useful in outlining the fungus cell wall. Of a number of methods described for the histochemical detection of polysaccharides, the periodic acid-Schiff stain was found to be by far the most practical and satisfactory.[1–3] This latter technique is also known as the Hotchkiss-McManus stain. The dye used is basic fuchsin. This red dye can be decolorized by the addition of sulfurous acid. The noncolored form of basic fuchsin is known as leuco-fuchsin or the Schiff reagent. The leuco form is unstable and, in the presence of various chemical groupings, particularly aldehydes, it is reconverted to the red form; *i.e.*, there is a reconstitution of the basic fuchsin to its original red color.

Ordinarily, tissues do not contain sufficient aldehyde groupings to recolorize the Schiff reagent. However, periodic acid has the property of converting complex polysaccharides (polyhydroxides or polyglycols) into the corresponding polyaldehydes; *i.e.*, periodic acid will bring about a hydrolysis and oxidation of cellulose and chitin so as to liberate aldehyde groupings which are Schiff positive. A Schiff-positive material is one which will recolorize the Schiff reagent. There are, thus, two essential steps in this procedure: (1) the release of aldehydes from cellulose-chitin complexes in the fungus wall by periodic acid; (2) exposure of the tissue to the Schiff reagent, with reconstitution to the original red color resulting from the combination of the aldehydes with the Schiff reagent.

The method of staining hyphae in skin scrapings is simple and quick. It thus becomes practical as a routine clinical procedure for the diagnosis of superficial fungus infections. The procedure for staining biopsy specimens is somewhat more elaborate but basically similar and practical.

Method for Staining Skin Scrapings. Use egg albumen as an adhering agent for the scrapings. Avoid too thick smears, and make nail and scrapings of horny material as fine as possible. Preferably, a drop or two of albumen should be placed directly on the lesion, which is then scraped with a knife. The scrapings are then smeared over the slide.

(1) Immerse slide in 95 per cent alcohol for one minute.

(2) Immerse in a 5 per cent aqueous solution of periodic acid for three minutes.

(3) Wash in running water for two minutes.

(4) Stain in Schiff reagent for 8 minutes.

(5) Rinse in running water for one minute.

(6) Dehydrate through 95 per cent alcohol, absolute alcohol, and xylol.

(7) Mount with clarite.

(The last two steps may be omitted if permanent mounts are not required. Simply mount in a non-acid containing water soluble mounting medium after step 5.)

Method for Staining Tissue Sections. Tissue may be processed through any routine fixative, embedded, sectioned, and dehydrated.

(1) Deparaffinize in xylol. Rinse in absolute alcohol. Rinse in distilled water.

(2) Immerse in a 1 per cent solution of periodic acid for five minutes.

(3) Wash under tap water for five minutes.

(4) Transfer to Schiff reagent for ten minutes.

(5) Wash under tap for ten minutes.

(6) Dehydrate, clear, and mount.

Preparation of Schiff Reagent. Dissolve 0.5 gram of basic fuchsin by pouring over it 100 cc. of boiling distilled water. Cool to 50°C. Filter and add 10 cc. of 1 normal hydrochloric acid and 0.5 gram potassium metabisulphite to filtrate. Allow solution to stand in the dark overnight. The solution should be colorless or pale straw colored. To decolorize completely, add 0.25 to 0.50 gram activated charcoal, shake thoroughly, and filter immediately. Keep in tightly stoppered bottle in refrigerator. This can be used until color reappears.

Results. The fungus cells stain red or various shades of magenta. The tissue background is colorless or slightly pink. In short, excellent contrast is afforded and a moment's glance under the low power microscope is sufficient to locate the fungus. In superficial ringworm infections, more positive diagnoses are possible than with the potassium hydroxide technique. Small hyphal fragments are easily detected and the problem of artifacts is completely eliminated. Moreover, the slide may be made a permanent one which can be put away and examined at convenience or filed. As with the potassium hydroxide technique, the various species of ringworm cannot be identified with this stain.

The method has proved especially valuable for the detection of fungi invading the viscera (the systemic mycoses). The staining is so brilliant that even the nonskilled and those who have no acquaintance with fungus diseases are able to recognize specifically the causative fungus.

It has become evident from preliminary studies that many of our previous concepts concerning the morphology of these organisms in tissue were erroneous. For instance, the "capsule" of *Histoplasma capsulatum* so clearly seen in the hematoxylin and eosin stain seems most certainly to be an artifact when stained by the Hotchkiss-McManus technique. The organisms appear much larger because the wall is clearly defined and seems to lie outside the so-called "capsule" seen in the hematoxylin and eosin sections. Evidently, the cell wall was not visualized by the previous techniques and the "capsule" was simply part of the unstained cytoplasm. *Histoplasma capsulatum* would, therefore, seem to be poorly named.

Sporotrichum schenckii is another organism which often appears to have a capsule in tissue. This capsule is also an artifact as shown by the Hotchkiss-McManus technique. A point of some practical consideration is that *Sporotrichum* cells are exceedingly difficult to visualize in human tissue, although paradoxically culture is easy. The organisms are scarce. In the sections of human sporotrichosis we have had the opportunity to examine by the periodic acid-Schiff technique, it has always been possible to locate and identify a few fungus cells, whereas other staining methods generally failed.

It is interesting to note that the large capsules of *Cryptococcus neoformans* also stain with this procedure. This supports the thesis that the capsule is made up of hyaluronic acid. Hyaluronic acid is a normal component of tissue. Since it is a mucopolysaccharide, its acceptance of the stain is understandable.

Although the studies are in the preliminary stage, it has become quite clear that much new information will be gained by the use of this technique. A more precise description of the morphology of these organisms is now possible. The experimental study of the systemic mycoses is greatly facilitated. It also seems that the diagnosis of fungus disease will no longer require specialized knowledge and that this procedure will become a diagnostic method in the routine laboratory.

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